Release of glutathione from erythrocytes and other markers of oxidative stress in carbon monoxide poisoning

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Thom, Stephen R., Melissa Kang, Donald Fisher, and Harry Ischiropoulos. Release of glutathione from erythrocytes and other markers of oxidative stress in carbon monoxide poisoning. J. Appl. Physiol. 82(5): 1424–1432, 1997.—Rats exposed to CO in a manner known to cause oxidative stress in brain exhibited a twofold increase in plasma levels of oxidized proteins, thiobarbituric acid-reactive substances (TBARS), oxidized glutathione (GSSG), and reduced glutathione (GSH). Changes were neither directly related to hypoxic stress from carboxyhemoglobin nor significantly influenced by circulating platelets or neutrophils. Treatment with the nitric oxide synthase inhibitor N-nitro-L-arginine methyl ester inhibited elevations in GSH and GSSG but not changes in oxidized proteins or TBARS, suggesting that two oxidative mechanisms may be operating in this model and that GSH and GSSG elevations involved nitric oxide-derived oxidants. Elevations of blood GSH and GSSG occurred at different anatomic sites, indicating that no single organ was the source of the increased peptides. Animals that underwent exchange transfusion with a hemoglobin-containing saline solution did not exhibit elevations in GSH and GSSG, suggesting that blood-borne cells released these peptides in response to oxidative stress. In in vitro studies, erythrocytes, but not platelets and leukocytes, responded to oxidative stress from peroxynitrite by releasing GSH, whereas no release was observed in response to nitric oxide or superoxide. Glucose, maltose, and cytochalasin B agents that protect extracellular components of the hexose transport protein complex from oxidative stress, prevented GSH release. The data indicate that nitric oxide-derived oxidants are involved in CO-mediated oxidative stress within the vascular compartment and that elevations of several compounds may be useful for identifying exposures to CO likely to precipitate brain injury.

Peroxynitrite; nitric oxide; oxidized proteins; thiobarbituric acid reactive substances; hexose transport

OXIDATIVE STRESS in the body occurs when the production of free radicals overwhelms the antioxidant defense systems, and oxidative damage of cells is the result. In recent years, reductions in the level of plasma antioxidants such as vitamin E, as well as increases in oxidized glutathione (GSSG) and products of lipid peroxidation, have been used as "biomarkers" of oxidative injury (15, 21, 22, 24, 37). These changes can provide insights into pathophysiology, and, in a clinical situation, they may be useful to assess the severity of an injury.

Clinical observations suggest that the pathophysiology of CO poisoning is not based solely on the hypoxic stress mediated by an elevation in the blood carboxyhemoglobin (HbCO) level (11). In particular, the risk of permanent brain injury appears to be higher when CO poisoning involves a pattern in which the exposure occurs over a span of time, referred to as a "soak," and then an individual suffers an interval of unconsciousness, presumably due to a further elevation in the environmental CO level (6, 20). Unfortunately, the pattern of CO poisoning that a patient has suffered is not always clear in an emergency setting, and there are currently no reliable laboratory tests that predict which exposures are likely to precipitate neurological injuries.

We have found that several different oxidative processes occur in association with CO poisoning in a rat model. Perivascular oxidative changes mediated by nitric oxide (NO) are indicated by deposits of nitrotyrosine, and these changes occur even when exposures last for only relatively brief time periods of <1 h (13). A more extensive process of brain lipid peroxidation occurs when exposure to CO follows a pattern similar to that seen among patients at high risk for developing brain injuries (29). This pattern involves exposing rats to 1,000 parts/million (ppm) of CO for 40 min as the soak and then exposure to CO at 3,000 ppm, which eventually causes hypotension and cerebral hypoperfusion due to cardiac dysfunction. We have found that cerebral hypoperfusion is manifested grossly as unconsciousness (17, 29). After rats are exposed to CO, a cascade of cellular and biochemical events occurs. Vascular oxidative changes attributable to NO contribute to leukocyte attachment to the brain microvasculature; these leukocytes become activated and cause conversion of endothelial xanthine dehydrogenase to oxidase; and the oxygen radicals that are generated cause brain lipid peroxidation (13, 29–31). Others have poisoned rats with CO according to a similar pattern and found functional neurological impairments and histopathology in the hippocampus (35).

The aim of this study was to evaluate whether there may be plasma markers of oxidative stress in rats that have been exposed to CO. Changes in the concentration of reduced glutathione (GSH) and GSSG in plasma led to further investigations, including a study of the effect of peroxynitrite on isolated red blood cells (RBC). Peroxynitrite is a strong oxidant that is generated by the near-diffusion limited reaction between superoxide and NO (12). Production of both superoxide and NO is increased during CO poisoning (13, 33, 38). RBC have been shown to liberate GSSG in response to oxidative stress, and reducing equivalents from extracellular GSH can be transduced across the cell membrane via the hexose transport protein complex (7, 16). However,
 MATERIALS AND METHODS

Animals and reagents. Wistar male rats (Charles River Laboratories, Wilmington, MA) weighing 200–290 g were fed a standard diet and water ad libitum. Diethylamine NONOate (DENO), 3-morpholinosyndnonimine (SIN-1), and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (carboxy-PTIO) were purchased from Cayman Chemical (Ann Arbor, MI). Unless otherwise specified, all other reagents were purchased from Sigma Chemical (St. Louis, MO).

Exposure of rats to CO. Exposure to CO followed our model described in detail in previous publications (13, 17, 29–31). In brief, rats were placed in a 7-liter Plexiglas chamber into which a mixture of 1,000 ppm CO in air was flushed through at a rate of 8–12 l/min. At the start of the exposure, a small volume of pure CO was injected into the gas stream to compensate for the volume of air already in the chamber. This procedure allowed the rats to be exposed to 1,000 ppm CO from the very beginning of the study. Rats were exposed to 1,000 ppm CO for 60 min, at which time the gas was switched to 3,000 ppm CO in air and another CO bolus was added to rapidly achieve the 3,000 ppm concentration. Rats usually lost consciousness within 20 min and were removed from the chamber to breathe room air. In preliminary studies, we have found that all rats will lose consciousness if exposed to 3,000 ppm CO for up to 40 min. However, we have found that the duration of exposure as well as the concentration of CO used influences perivascular oxidative stress in this model (13). Therefore, to keep rats matched as closely as possible over the course of this investigation, those that did not spontaneously lose consciousness within 20 min were rendered unconscious with a 60-cc bolus of pure CO. This was necessary with approximately one in three rats.

We have found that, whether unconsciousness occurs with exposure to 3,000 ppm CO or with the bolus of pure CO, it is a reliable outward manifestation of systemic hypotension and cerebral hypoperfusion and that the biochemical cascade causing global oxidative stress is the same with either method of inducing hypotension and unconsciousness (17, 29–31). In some studies, rats were exposed to only 1,000 ppm CO for 60 min with no bolus of CO to cause unconsciousness or to 3,000 ppm CO for just 1–2 min plus a bolus of pure CO to rapidly cause unconsciousness. In other studies, rats were made thrombocytopenic by an intraperitoneal injection of 1.5 ml/kg rat plasma-adsorbed rabbit anti-rat platelet antiserum (Inter-cell Technologies, Hopewell, NJ) given 2 h before exposure to CO. Neutropenia was caused by an intraperitoneal injection of 4 ml/kg antineutrophil antiserum (Inter-cell Technologies, Hopewell, NJ) given 2 h before study. In preliminary studies, we have found that all rats will lose consciousness if exposed to 3,000 ppm CO for up to 40 min. However, we have found that the duration of exposure as well as the concentration of CO used influences perivascular oxidative stress in this model (13). Therefore, to keep rats matched as closely as possible over the course of this investigation, those that did not spontaneously lose consciousness within 20 min were rendered unconscious with a 60-cc bolus of pure CO. This was necessary with approximately one in three rats.

In vitro assays. RBC were separated by centrifuging heparinized blood underlayered with Histopaque, as described above. The RBC layer was removed and combined in a ratio of 1:2 with buffer (100 mM potassium phosphate, 0.9% NaCl, and 0.1 mM diethylenetriaminepentaacetic acid, pH 7.4). Leukocytes and platelets used for GSH- and GSSG-leakage studies were initially prepared simply by centrifuging heparinized blood and removing the supernatant anduffy coat. Cells were combined with a volume of buffer equal to the initial blood volume, and assays were performed with free radical generators. When no GSH or GSSG leakage was measured, we repeated our studies with more concentrated suspensions of cells prepared from the upper layers of isolated RBC preparations. The platelet- and leukocyte-containing bands were obtained after 12 ml of blood were centrifuged with the use of Histopaque. The layers were combined with 8 ml buffer and centrifuged at 700 g for 10 min, and then the pellet was resuspended in 5 ml of buffer. A total of five studies were performed with suspensions of leukocytes and platelets prepared with an average of 18,240 ± 2,920 leukocytes and 0.7 ± 0.3 × 10⁶ platelets/μl.
Both the RBC and leukocyte-platelet preparations were incubated at room temperature (25°C) with free radical generators. The concentration of GSH and GSSG in the leukocyte-platelet suspensions was assayed every 5 min. As outlined in RESULTS, a rather complex pattern was observed with RBC preparations, and the initial rates of release of GSH and GSSG were taken as the change in concentration over the first 3 min of incubation. Stock solutions of SIN-1 were prepared in 50 mM phosphate buffer, pH 5.0, and solutions of DE NO were prepared in 50 mM phosphate buffer, pH 8.5. Superoxide radical was generated in solutions containing 4 mM hypoxanthine and xanthine oxidase (0.017 U/ml). The rate of peroxynitrite formation from SIN-1 was assessed by measuring the oxidation of dihydrorhodamine 123 to rhodamine, following published methods (14). The half-life of DE NO was determined based on its characteristic ultraviolet absorption spectrum (E\text{250} = 6,500), and liberation of NO was measured directly with a NO-selective polarographic probe (ISO-NO, World Precision Instruments, Sarasota, FL). The rate of superoxide generation was assessed as the superoxide dismutase-inhibitable rate of cytochrome c reduction (18).

Statistics. Statistical significance was determined by analysis of variance followed by Scheffe’s test (26). The level of significance was taken as P < 0.05. Results are expressed as means ± SE.

RESULTS
Plasma indicators in different patterns of CO-induced oxidative stress. Concentrations of GSSG, DNPH-reactive oxidized proteins, and TBARS were increased approximately twofold over control after rats were poisoned with CO according to the standard model that causes brain lipid peroxidation (Fig. 1). However, if rats were exposed to CO according to different patterns that raise the HbCO level but do not cause brain lipid peroxidation (29, 30), no increase in oxidized products was observed (Fig. 1). The mean HbCO level in rats poisoned according to the standard model was 63 ± 3% (n = 24). When rats were exposed to a bolus of CO to cause unconsciousness in <2 min, the mean HbCO level was 81 ± 1% (n = 5), a level significantly higher than in rats exposed to the standard model. The mean HbCO level in rats exposed to 1,000 ppm CO for 1 h was 51 ± 2% (n = 7), insignificantly different from the level in rats exposed to the standard model. We conclude from these findings that mechanisms in addition to the hypoxic stress precipitated by CO were involved with the elevation in oxidized products seen with the standard CO model.

Influence of platelets and neutrophils on plasma indicators of CO-induced oxidative stress. Platelets liberate relatively large amounts of NO during CO poisoning (33). Because platelet-derived NO does contribute to perivascular oxidative stress (13), we evaluated whether oxidized products in plasma were elevated in thrombocytopenic rats (Fig. 1). Elevated levels of GSSG, TBARS, and oxidized proteins were found in rats with circulating platelet counts that were only 16 ± 8% (n = 6) of the control level (Fig. 1). We conclude that platelets do not appear to contribute significantly to the changes observed in plasma.

Because neutrophils are involved in brain oxidative changes that are detected ~1.5 h after CO poisoning (31), we also examined plasma indicators in rats made neutropenic by injections of antineutrophil antiserum. Neutropenia (neutrophil count <200 cells/µl blood) did not alter elevations in plasma indicators in response to CO poisoning (Fig. 1).

Plasma GSH is increased in CO poisoning. The ratio of GSSG to GSH in plasma is often a sensitive indicator of systemic oxidative stress (9, 21). The GSSG-GSH ratio in control rats was 0.026 ± 0.005 (n = 14), and the level was significantly different in rats exposed to the standard CO model (0.030 ± 0.005; n = 15). We found that, in rats after CO poisoning, this ratio remained unchanged because the concentration of GSH in plasma also doubled (Table 1). Our initial hypothesis for the rise in both GSH and GSSG was that hemolysis may...
can be inhibited by injecting rats with the nitric oxide perivascular oxidativestress (13, 32, 33). These changes during CO poisoning, and

METHODS, plasma TBARS and DNPH-reactive proteins pretreated with L-NAME as described in MATERIALS AND

Table 1. Plasma glutathione levels

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>GSH, µM</th>
<th>GSSG, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21</td>
<td>13.8 ± 0.8</td>
<td>0.33 ± 0.07</td>
</tr>
<tr>
<td>CO, 1,000 ppm × 1 h</td>
<td>6</td>
<td>15.0 ± 1.5</td>
<td>0.35 ± 0.14</td>
</tr>
<tr>
<td>CO bolus, &lt;2 min</td>
<td>3</td>
<td>14.9 ± 3.2</td>
<td>0.18 ± 0.08</td>
</tr>
<tr>
<td>CO, standard model</td>
<td>24</td>
<td>28.7 ± 2.6*</td>
<td>0.94 ± 0.20*</td>
</tr>
<tr>
<td>Standard model, low platelets</td>
<td>6</td>
<td>40.7 ± 6.5*</td>
<td>0.86 ± 0.29*</td>
</tr>
<tr>
<td>Standard model, neutropenia</td>
<td>6</td>
<td>36.6 ± 4.7*</td>
<td>0.97 ± 0.16*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Heparinized blood was obtained from rats exposed to CO according to different patterns and assayed for reduced glutathione (GSH) and oxidized glutathione (GSSG) as described in MATERIALS AND METHODS. Some rats were exposed to 1,000 ppm CO for 1 h or to a bolus of CO to cause unconsciousness in <2 min. Other rats were exposed to standard model of CO poisoning (see MATERIALS AND METHODS) without other manipulations after circulating platelet count was decreased to 16 ± 8% of control and after neutrophil count was decreased to < 200 cells/µl blood; n, no. of rats studied. *P < 0.05. Concentrations of GSH in thrombocytopenic or neutropenic rats were not statistically different from those in rats exposed to standard CO model.

...have occurred. To evaluate this possibility, we measured the free Hb and potassium concentrations in plasma. No change in either parameter was found after CO poisoning. The free Hb concentration was 3.6 ± 1.0 mg/100 ml plasma (n = 8) in control rats and 3.0 ± 0.5 mg/100 ml (n = 8) in rats poisoned with CO. The plasma potassium concentration was 4.4 ± 0.3 meq/l (n = 8) in control rats and 4.4 ± 0.3 meq/l (n = 11) after CO poisoning. We conclude from these data that the elevations in GSH and GSSG were not due to overt RBC damage that caused hemolysis.

...Elevations in plasma GSH followed the same pattern as that seen for TBARS, DNPH reactive proteins, and GSSG. Hence, no significant increases were found in rats exposed to 1,000 ppm CO for 1 h or to a bolus of CO to cause unconsciousness in <2 min (Table 1). Elevations in GSH did occur when thrombocytopenic or neutropenic rats were poisoned with CO.

...Effects of L-NAME treatment. Platelets as well as other cell sources produce increased amounts of NO during CO poisoning, and NO-derived oxidants cause perivascular oxidative stress (13, 32, 33). These changes can be inhibited by injecting rats with the nitric oxide synthase inhibitor L-NAME (13, 33). When rats were pretreated with L-NAME as described in MATERIALS AND METHODS, plasma TBARS and DNPH-reactive proteins were still elevated after standard CO poisoning, but plasma GSH and GSSG levels were not significantly increased over the control level. Thus, in rats injected with L-NAME and then poisoned with CO, the plasma TBARS concentration was 4.9 ± 0.7 µM (n = 4; P < 0.05 vs. control) and the DNPH-reactive protein concentration was 1.7 ± 0.4 nmol/mg protein (n = 4; P < 0.05). However, the GSH level was 19.8 ± 0.9 µM (n = 8; not significant vs. control), and the GSSG level was 0.40 ± 0.10 µM (n = 8; not significant).

...GSH and GSSG levels in blood from different anatomical sites. There is an active cycle of glutathione transfer in the body. The liver is a major site for production and release of GSH into the plasma, and GSH is removed from the plasma by the kidneys (5, 10). To investigate whether CO caused a disturbance in this pattern, GSH and GSSG levels were measured in blood taken from different vessels of both control rats and rats immediately after they were exposed to the standard CO model (Table 2). In control rats, the glutathione blood levels decreased in the following order: aorta, vena cava proximal to the hepatic vein, distal vena cava, and then renal vein. After CO poisoning, the same general pattern was found, except that the levels were all significantly higher than in control rats. We conclude that no single organ (e.g., liver) was the source for the increased concentrations of GSH and GSSG after CO poisoning.

Table 2. Plasma glutathione levels from different anatomical sites

<table>
<thead>
<tr>
<th>Blood Vessel</th>
<th>Control</th>
<th>CO</th>
<th>Control</th>
<th>CO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta</td>
<td>19.0 ± 2.0 (6)</td>
<td>28.3 ± 2.9* (7)</td>
<td>0.41 ± 0.04 (7)</td>
<td>0.70 ± 0.13* (5)</td>
</tr>
<tr>
<td>Proximal vena cava</td>
<td>12.2 ± 2.0 (4)</td>
<td>26.5 ± 4.3* (3)</td>
<td>0.12 ± 0.05 (4)</td>
<td>0.23 ± 0.03* (3)</td>
</tr>
<tr>
<td>Distal vena cava</td>
<td>7.4 ± 0.9 (7)</td>
<td>21.4 ± 2.5* (8)</td>
<td>0.15 ± 0.06 (7)</td>
<td>0.22 ± 0.09 (5)</td>
</tr>
<tr>
<td>Renal vein</td>
<td>3.6 ± 0.6 (5)</td>
<td>9.8 ± 2.5* (5)</td>
<td>0.002 ± 0.002 (5)</td>
<td>0.07 ± 0.04* (5)</td>
</tr>
</tbody>
</table>

Values are means ± SE. Nos. in parentheses, no. of rats assayed. Heparinized blood was obtained from control rats or rats that had been exposed to standard CO model. Proximal vena cava is that portion between hepatic vein and heart. Blood from distal vena cava was obtained from the abdominal segment of vena cava, distal to hepatic vein. *P < 0.05 vs. control value from same blood vessel.
Table 3. Arterial blood-gas results from rats after exchange transfusion

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>pH</th>
<th>Pco2, Torr</th>
<th>Pco2, Torr</th>
<th>HbCO, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>After transfusion</td>
<td>6</td>
<td>7.39 ± 0.02</td>
<td>41 ± 2</td>
<td>92 ± 2</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Air (Control)</td>
<td>3</td>
<td>7.43 ± 0.02</td>
<td>28 ± 1</td>
<td>121 ± 3</td>
<td>0.8 ± 0.9</td>
</tr>
<tr>
<td>CO</td>
<td>3</td>
<td>7.06 ± 0.3</td>
<td>10 ± 0.6</td>
<td>121 ± 7</td>
<td>63 ± 2</td>
</tr>
</tbody>
</table>

Values are means ± SE. Arterial blood-gas samples were obtained from 6 rats immediately after completion of exchange transfusion, and 1 h later in rats exposed to air (control group) or to CO. All values in CO group except partial pressure of O2 (PO2) are significantly different from control. Pco2, partial pressure of CO2; HbCO, carboxyhemoglobin.

The CO-exposed group, and a mild respiratory alkalosis in the control group by the end of the study.

The values for plasma markers of oxidative stress in the rats after completing the exchange transfusion, and then 1 h later in rats exposed either to air (control) or CO, are shown in Table 4. The results are notable for a lack of change in GSH and GSSG after CO poisoning. There was a significant elevation in DNHPh-reactive proteins in the CO group. However, the values measured, even in rats immediately posttransfusion and before CO exposure, were fivefold greater than what was typically found in rats that had not undergone exchange transfusion. Oxidation of Hb and other proteins in the process of preparing the exchange transfusion solution is a likely source of these oxidized proteins. There was a slight, but statistically insignificant, elevation in plasma TBARS in the CO group after exchange transfusion. It should be noted, however, that the TBARS level in all groups was sixfold lower than in control rats that had not undergone exchange transfusion.

We conclude from these studies that circulating cells were likely to be the source of the GSH and GSSG that caused the elevation in plasma levels seen after CO poisoning. The values for TBARS and DNHPh-reactive proteins could not easily be interpreted because of artifacts of the procedure.

Glutathione leakage from RBC, leukocytes, and platelets. Because plasma GSH and GSSG were not elevated in CO-poisoned rats pretreated with l-NAME, we hypothesized that NO or the NO-derived oxidant peroxynitrite may have been responsible for precipitating GSH or GSSG release from RBC or other circulating cells. A series of studies was carried out with RBC and leukocyte/platelet preparations (see MATERIALS AND METHODS) in vitro to test this hypothesis.

Table 4. Plasma markers of oxidative stress in rats after exchange transfusion

<table>
<thead>
<tr>
<th>Group</th>
<th>After Transfusion</th>
<th>Air Group</th>
<th>CO Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH, µM</td>
<td>15 ± 2</td>
<td>14 ± 2</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>GSSG, µM</td>
<td>1.5 ± 0.5</td>
<td>0.4 ± 0.1</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>DNHPh-R, nmol/mg</td>
<td>3.7 ± 0.1</td>
<td>4.0 ± 0.2</td>
<td>5.4 ± 0.5*</td>
</tr>
<tr>
<td>TBARS, µM</td>
<td>0.29 ± 0.02</td>
<td>0.29 ± 0.01</td>
<td>0.33 ± 0.02</td>
</tr>
</tbody>
</table>

Values are means ± SE. Plasma samples were obtained from 12 rats immediately after completion of the exchange transfusion, and 1 h later in rats exposed to air (control group n = 6) or to CO (n = 6). DNHPh-R, dinitrophenylhydrazine-reactive; TBARS, thiobarbituric acid-reactive substances. *P < 0.05.

When RBC were incubated with SIN-1, which generates peroxynitrite by spontaneously releasing both superoxide and ·NO, there was a dose-dependent increase in the initial rate of release of GSH (Table 5). In individual experiments, the rate of GSH release was influenced by the number of RBC in the suspension. Therefore, to compare the results of daily experiments, rates of GSH release were normalized to the Hb concentration of the preparations. In day-to-day studies, the mean Hb concentration in the suspensions was 6.3 ± 0.3 g Hb/dl (n = 30). The rise in GSH caused by SIN-1 was not caused by hemolysis. No increase in buffer potassium concentration or in free Hb was measurable even in 20 min when cells were incubated with 333 µM SIN-1.

Release of GSH and GSSG from concentrated suspensions of leukocytes and platelets (see MATERIALS AND METHODS) was also evaluated. There was no detectable release of GSH or GSSG when cells were incubated with 333 µM SIN-1 for up to 40 min in five separate trials.

SIN-1 decomposes to simultaneously generate superoxide, ·NO, and the stable decomposition product SIN-1 C. Peroxynitrite is generated by the diffusion-limited reaction between superoxide and ·NO. When RBC preparations were incubated with 111 µM SIN-1 in combination with 100 µM carboxy-PTIO, a potent scavenger of ·NO (3), the rate of GSH release from RBC was 3.4 ± 2.1 µmol·g Hb·min⁻¹·min⁻¹ (n = 5), only 15% of the expected rate caused by 111 µM SIN-1, as listed in Table 5 (P < 0.05). Incubation of 111 µM SIN-1 in combination with 1 mg/ml superoxide dismutase, to scavenge superoxide liberated by SIN-1, resulted in a rate of only 1.9 ± 1.8 µmol GSH·g Hb⁻¹·min⁻¹·min⁻¹ (n = 4), significantly less than the rate with SIN-1 by itself (P < 0.05).

Table 5. Rate of GSH and GSSG release from isolated RBC incubated with SIN-1

<table>
<thead>
<tr>
<th>SIN-1, µM</th>
<th>GSH Release</th>
<th>GSSG Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 ± 0.08(8)</td>
<td>0.21 ± 0.18(8)</td>
</tr>
<tr>
<td>6</td>
<td>5.9 ± 1.7(3)</td>
<td>0 ± 0(3)</td>
</tr>
<tr>
<td>33</td>
<td>7.6 ± 0.2(3)</td>
<td>0 ± 0(4)</td>
</tr>
<tr>
<td>55</td>
<td>17.8 ± 1.1(4)</td>
<td>0.26 ± 0.07(7)</td>
</tr>
<tr>
<td>111</td>
<td>22.5 ± 4.4(5)</td>
<td>0.56 ± 0.17(7)</td>
</tr>
<tr>
<td>333</td>
<td>37.9 ± 10.2(6)</td>
<td>0.56 ± 0.19(9)</td>
</tr>
</tbody>
</table>

Values are means ± SE of rate of release (µmol·g Hb⁻¹·min⁻¹·min⁻¹) from red blood cells (RBC) in the first 3 min of incubation with 3-morpholinosydnonimine (SIN-1). RBC were isolated from heparinized blood, suspended in buffer, and exposed to concentrations of SIN-1, as outlined in MATERIALS AND METHODS. Nos. in parentheses, no. of trials with blood from different rats. *P < 0.05.
compound that liberates SIN-1 caused the release of GSH and GSSG from RBC.

RBC preparations were also incubated with DENO, a compound that liberates NO at physiological pH. At pH 7.4 and room temperature the half-life of DENO was 2.4 min, and 6.5 µM DENO generated NO at a rate of 1.8 µmol/min (see MATERIALS AND METHODS). When RBC were exposed to this flux, the initial rate of GSH release was 1.4 ± 1.4 µmol·g Hb⁻¹·min⁻¹ (n = 4), which was not significantly different from control but was significantly different from samples incubated with 111 µM SIN-1.

The effects of superoxide radical and H₂O₂ on GSH release were also assessed. A preparation of 4 mM hypoxanthine plus xanthine oxidase generated superoxide at 2.3 µmol/min (see MATERIALS AND METHODS). When RBC were exposed to this flux, the initial rate of GSH release was 0 ± 0 µmol GSH·g Hb⁻¹·min⁻¹; n = 4.

We found that the initial rate of GSSG release from RBC was small and not significantly affected by SIN-1 (Table 5). However, if incubations were lengthened, a prominent increase was found in the concentration of GSSG in the suspension. This effect is illustrated in Fig. 2, where temporal changes are shown for GSH and GSSG concentrations in RBC suspensions exposed to 111 µM SIN-1. The concentration of GSSG in control suspensions of RBC incubated in buffer for 60 min was 12.6 ± 6.9 µmol/g Hb (n = 5), in contrast to the GSSG concentration at 60 min in the presence of 111 µM SIN-1 (129 ± 22 µmol/g Hb; n = 7; P < 0.05). Obviously, a large portion of the GSSG measured at this time may have been derived from the GSH previously released by the RBC, as well as GSSG directly released by RBC.

Effects of hexoses and cytochalasin B. A thiol-disulfide exchange mechanism exists in RBC that allows transduction of reducing equivalents into cells via sulfur-rich membrane proteins (8). Components of these proteins can be protected from some oxidants by a number of sugars and by cytochalasin B. We found that incubation with glucose, maltose, or cytochalasin B significantly reduced the flux of GSH from RBC exposed to 111 µM SIN-1 (Table 6). The effect of these agents was not caused by scavaging of peroxynitrite, based on assays conducted with 5 µM SIN-1 incubated with dihydrodihromadine 123. The rate of rhodamine 123 formation was monitored as an index for peroxynitrite-mediated reactions. The rate was 4.6 ± 0.3 nmol/min (n = 8) with no additions. In the presence of 1 µM cytochalasin, 5 mM glucose, or 5 mM maltose, the rates were 4.6 ± 0.2 (n = 3), 4.5 ± 0.4 (n = 3), and 4.6 ± 0.8 (n = 3), respectively (no significant differences from control).

**DISCUSSION**

Plasma concentrations of TBARS, oxidized proteins, GSH, and GSSG were significantly increased in rats exposed to CO in a manner known to cause brain oxidative stress. Among the oxidation products measured, glutathione values were unique in that elevations of GSH and GSSG were inhibited in rats pretreated with the nitric oxide synthase inhibitor L-NAME. These data suggest that two mechanisms of action may be operating to cause the elevations in plasma oxidized products. We were particularly interested in the role NO may play with glutathione elevations, because early perivascular oxidative changes during CO exposure are mediated by NO-derived oxidants, and these NO-mediated changes are required for the subsequent cascade of cellular and biochemical changes that lead to brain lipid peroxidation after CO poisoning (13).

Plasma glutathione concentration has been found to be the highest in the hepatic vein (5). We attempted to measure plasma levels by aspirating blood from this vessel. However, the space between the diaphragm and the dome of the liver, where the hepatic vein enters the vena cava, is quite small in the rat. The blood we obtained from this area may therefore be considered to be a mixture of blood from the hepatic vein and the proximal vena cava. We found that the GSH concentration was slightly higher in this blood vs. blood in the distal vena cava, but the value in aortic blood was still higher. We interpret these findings as indicative of hepatic production of GSH. However, other cells, possi-

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**Table 6.** Effects of hexoses and cytochalasin B on rate of GSH release from RBC exposed to 111 µM SIN-1

<table>
<thead>
<tr>
<th>Additions</th>
<th>GSH Release (µmol·g Hb⁻¹·min⁻¹)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>23.0 ± 1.1</td>
<td>7</td>
</tr>
<tr>
<td>Cytochalasin B, 1 µM</td>
<td>5.2 ± 0.8*</td>
<td>4</td>
</tr>
<tr>
<td>Glucose, 5 mM</td>
<td>2.1 ± 1.4*</td>
<td>7</td>
</tr>
<tr>
<td>Maltose, 5 mM</td>
<td>6.9 ± 3.0*</td>
<td>7</td>
</tr>
</tbody>
</table>

Values are means ± SE indicating rate of GSH release (µmol·g Hb⁻¹·min⁻¹) from RBC in first 3 min of incubation with 111 µM SIN-1; RBC were isolated from heparinized blood, suspended in buffer, and exposed to 111 µM SIN-1 in addition to agents shown. n, no. of trials with blood from different rats. *P < 0.05.
ly in the lungs, also release significant amounts of GSH in the rat. The pertinent issue in relation to our study is that the GSH level in blood obtained from all vessels was significantly greater after CO poisoning. Hence, no single organ could be identified as the source for the increase in plasma glutathione found after CO poisoning.

Elevations in plasma oxidized proteins, TBARS, and glutathione did not correlate with HbCO levels, which were similar in all three patterns of CO exposure shown in Fig. 1. Elevations in plasma markers only occurred when rats were exposed to CO according to the pattern that causes brain lipid peroxidation (13, 29–31). We have found that during CO poisoning there is a precipitous increase in generation of -NO in association with unconsciousness and, therefore, during brain hypoperfusion (13, 17). It is likely that plasma markers of oxidative stress were detected after the standard CO exposure model and not the other patterns of CO poisoning, because the standard model of exposure causes the most intense oxidative stress.

RBC appear to be the source of the elevated plasma GSH and GSSG in CO poisoning. RBC release GSH in response to exposure to peroxynitrite but not in response to -NO or superoxide radical. This discrete response, which may be related to the chemical reactivity of peroxynitrite, may be the reason why the phenomenon has not been described in other studies of RBC oxidative stress. The sources for the oxidizing species during CO exposure remains unclear. Neutrophils and platelets do not seem to be involved, and the vascular endothelium is a strong candidate. We recently reported that the concentration of nitrotyrosine, a relatively specific marker of peroxynitrite formation in vivo, was significantly increased in the brains of CO-poisoned rats and that nitrotyrosine deposits were particularly intense surrounding cerebral blood vessels (13). We have also reported that cultured endothelial cells release both NO and peroxynitrite in association with exposure to CO (32). Interestingly, we have been unable to measure significant release of GSH or GSSG from cultured vascular endothelial cells exposed to CO (unpublished observation).

RBC have a high antioxidant capacity, and they are able to scavenge both internal oxidants generated by Hb autooxidation and the partially reduced oxygen species that diffuse across the RBC membrane from extracellular sources (36). This is achieved through the combined activities of superoxide dismutase, catalase, and the GSH-dependent redox cycle (28). Transport of GSH to the extracellular space is a property shared by many cell types, and it is thought to be a mechanism for transferring reducing compounds to the immediate environment of the cell to protect essential thiol groups on the membrane surface (19). Others have found GSH to be a facile agent for transducing reducing power both to and from the intracellular space in RBC (8, 25). However, in these studies, GSH per se did not traverse the cell membrane.

Reducing equivalents from GSH outside of RBC are transferred across the membrane to the intracellular space by a process involving thiol groups on the exterior membrane surface (7). Sulfur-containing constituents of the hexose-transport mechanism of RBC are located both inside and outside of the membrane as well as within the nonpolar interior of the membrane (1). We hypothesized that the hexose-transport complex may be involved with the transfer of GSH out of RBC in response to peroxynitrite-mediated oxidative stress. To test this hypothesis, RBC were incubated with either a transportable sugar (glucose), an impermeant sugar (maltose), or cytochalasin B. All three of these agents have been shown to protect extracellular sulfhydryls of the hexose-transport mechanism from oxidative stress (1). Because these agents inhibited peroxynitrite-mediated GSH release from RBC, we hypothesize that the hexose-transport complex is involved with GSH transfer.

The pattern for GSH and GSSG release from RBC, as depicted in Fig. 2, is likely to have resulted from the cumulative stress of peroxynitrite. Thus the initial release of GSH may be a defensive mechanism that occurred in response to oxidative attack on the sulfur-containing moieties of the membrane. However, as the flux of peroxynitrite was maintained over time, more intracellular GSSG accumulated while intracellular sulfhydryl targets were attacked. With this progressive rise in intracellular GSSG, the cells would be expected to begin pumping GSSG out to the surrounding medium. Several types of cells, including RBC, hepatocyte, and lens cells of the eye, transport GSSG out of the cell when the intracellular concentration is high, as for example during oxidative stress (4, 23, 27). This action has been theorized to be an “emergency” mechanism to rapidly reestablish thiol/disulfide homeostasis. Therefore, with regard to the pattern of GSH release from RBC, the GSSG measured in the extracellular space would be the combination of GSSG directly released by the cells as well as GSSG resulting from oxidation of the GSH initially pumped out. This process offers an explanation for why the extracellular concentration of GSSG after 60-min incubation with SIN-1 (see Fig. 2) was found to exceed the highest concentration of GSH that was measured.

It was surprising that we did not observe a change in the plasma GSSG-GSH ratio after CO poisoning, as the GSH released by RBC would eventually become oxidized. The kidney plays a major role in clearance of plasma glutathione (5, 10), and renal clearance is likely to have prevented the GSSG-GSH ratio from being altered. When the GSH and GSSG concentrations in blood from the renal vein and aorta were compared (Table 2), renal vein GSH concentration was found to be 17% of the concentration found in blood from the aorta, whereas GSSG concentration in renal vein blood was only 0.5% of the level in aortic blood. This suggests that the relative uptake of GSSG by the kidney is greater than uptake of GSH, which would diminish the rise in plasma GSSG concentration relative to the amount of GSH released by RBC. However, the g-glutamyl cycle is still poorly understood, and this issue requires additional investigations, including measurements of uri-
nary glutathione and an analysis of renal γ-glutamyl transpeptidase activity.

The physiological significance of an elevation in plasma glutathione will also require additional study. It seems reasonable that this elevation may serve as a defensive response to oxidative stress. Further work is necessary to characterize the mechanism of GSH release by RBC. With regard to the CO model, an elevation of the plasma GSH level in response to oxidative stress is unusual and offers suggestive evidence for involvement of peroxynitrite. From a clinical perspective, if plasma levels of oxidized products could be shown to be correlated with development of neurologic morbidity in patients, this laboratory measurement would fill an important void in the emergency evaluation of CO-poisoning victims. Objective parameters could then be used to identify patients who should receive more advanced, aggressive treatment.

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REFERENCES


